International Journal of Medicine and Pharmaceutical Sciences (IJMPS)
ISSN(P): 2250-0049; ISSN(E): 2321-0095
Vol. 5, Issue 3, Jun 2015, 45-52

EXPRESSION OF HOMOGENOUS 40KD HUMAN RECOMBINANT ERYTHROPOIETIN FROM CHO CELLS – A REVIEW

MERCY DEVASAHAYAM

Centre for Transgenic Studies, Sam Higginbottom Institute of Agriculture Technology and Sciences, Naini, Allahabad, Uttar Pradesh, India

ABSTRACT

Human recombinant erythropoietin (rEPO) is used for the treatment of anemia and chronic kidney disease. The review deals with makinSg recombinant human erythropoietin from various expression systems. Human rEPO is made predominantly for therapeutic applications from CHO mammalian cells. rEPO secreted from CHO cells is incompletely glycosylated resulting in human rEPO glycosylation heterogeneity. The review describes the process to obtain completely glycosylated human rEPO at 40kd without any low molecular weight contaminants or without any glycosylation hetergeneity. This can be achieved by adding a glycophosphatidylinositol anchor signal sequence C terminal of the recombinant glycoprotein such as EPO for homogenous expression in mammalian cells such as CHO.

KEYWORDS: Anaemia, CHO Expression System, Glycoforms, *Pichia pastoris*, Recombinant Erythropoietin, Thy-1

INTRODUCTION

Human recombinant erythropoietin (rEPO) is a widely used for the treatment of anemia. Human rEPO represents one of the largest biopharmaceuticals markets and is produced from Chinese Hamster Ovary cells (CHO). Human rEPO is a glycoprotein with complex oligosaccahrides which is responsible for its therapeutic efficacy, *in vivo* biological activity and half-life. In various clinical trials the available erythropoietin stimulating agents while decreasing the rate of transfusions have shown an increase in thromboembolic events and mortality (Spivak et al., 2009).

Human rEPO is a 166 amino acid glycoprotein with one serine O-linked oligosaccharide (ser126) and 3 N-linked glycosylation sites (Asn 24,38 and 83). EPO with biantennary oligosaacharides has low *in vivo* biologic activity due to rapid clearance from the systemic circulation by renal handling (Misaizu et al., 1995). EPO with tertiary branched oligosaccharides is suggested to have a higher plasma level with an effective transfer to target organs and stimulation of erythroid progenitor cells (Misaizu et al., 1995). CHO cells derived secreted human rEPO is fully active *in vivo* (Sasaki et al., 1987). However rEPO expressed in CHO cells demonstrates glycosylation heterogeneity with molecular weight of 34-48kd (Yoon et al., 2001) or 32-38kd (Surabattula et al., 2011) by western blot. However EPO purified from human urine is of 39kd without any glycosylation heterogeneity (Miyake et al. 1977). This review deals with the process to obtain completetly glycosylated human rEPO without any low molecular weight glycosylation contaminants from CHO cells.

Human rEPO purification is classified into three types: the development of efficacious human rEPO molecules by protein engineering; improvement of host cells by genetic engineering- i.e., introducing humanising and human compatible enzymes; and culture condition optimization.

EXPRESSION OF HUMAN rEPO FROM CHO CELLS

The 39kd band of human recombinant erythropoietin expressed from CHO cells has been purified by fractionation from spent media using reverse phase chromatography (Sasaki et al., 1987). The article does not describe the oligosaccharide analysis of the various rEPO glycoforms expressed from CHO cells (Sasaki et al., 1987). The oligosaccharide analysis of the 39kd band alone shows the presence of biantennary, triantennary and teteranatennary oligosaccahrides. N-acetyl lactosamine repeats have been observed in both the triantennary and tetraantennary oligosaccharides (Sasaki et al., 1987). As is for all secreted glycoproteins from CHO cells, rEPO is expressed as a smear between 33 - 48kd (Yoon et al., 2001), 32-37kd (Surabattula et al., 2011) or 25-37kd (Zhang et al 2013). Though recombinant erythropoietin is produced in CHO cells the loss in quality control is 80% due to the incomplete glycosylation (Zoph D & Vergis G, 2002).

Expression of human rEPO from human hepatoma cell line (Huh-7) has been observed at a lower molecular weight of 34kd (Kauser et al., 2011). Expression of human rEPO as a fusion protein with the Fc part of human IgG resulted in a high molecular weight protein as a dimer (Schriebla et al., 2006).

EXPRESSION OF rEPO FROM MUTANT CELL LINES

The IdID mutant CHO cells are deficient in UDP-galactose (UDP-Gal) and UDPN-acetylgalactosamine (UDP-GalNAc) 4-epimerase activity. Recombinant proteins produced with only glucose as sugar source have truncated N-linked and no O-linked carbohydrate chains due to their inability to produce UDP-Gal or UDP-GalNAc from UDP-glucose or UDP-Nacetylglucosamine (Kingsley et al., 1986; Krieger et al., 1989). Human rEPO from IdID mutant CHO cells is expressed as a 30kd protein with only oligomannose structures (Wasley et al., 1991). To reverse the effect of the mutant cells when rEPO was expressed in the presence of Gal alone or with GalNAc, rEPO was expressed as a glycoform smear between 32-42kd (Wasley et al., 1991). N-glycanase treatment of this 32-42kd smear resulted in a 21kd rEPO band with the O-linked oligosaccharide (Wasley et al., 1991).

ROLE OF OLIGOSACCAHARIDES IN EPO BIOLOGICAL ACTIVITY

It has been shown in rats that rEPO with tetrantennary oligosaccharides are more resistant to degradation that rEPO with biantennary oligosaccharides (Misaizu et al., 1995). rEPO with biantennary oligosaccharides disappeared from the plasma more quickly than rEPO with tetrantennary oligosaccharides (Misaizu et al 1995). Whole rat body autoradiography after 20 minutes of administration showed rEPO with biantennary oligosaccharides levels higher in the kidney rEPO than of tetrantennary oligosaccharides (Misaizu et al., 1995). Low *in vivo* biological activity was observed for rEPO with biantennary oligosaccharides than rEPO with tetrantennary oligosaccharides (Misaizu et al., 1995). rEPO with tetrantennary oligosaccharides is required for high plasma levels to stimulate erythroid progenitor cells in target organs such as liver, spleen, kidney and bone marrow (Misaizu et al., 1995).

HUMAN rEPO FROM TRANSGENIC ANIMALS

Human recombinant erythropoietin has been expressed in the milk of transgenic rabbit and mice. The human rEPO has been expressed as a C-terminal fusion protein with bovine b-lactoglobulin (Korhonen et al., 1997). Biological activity of the fusion protein was less than 15% of standard human rEPO (Korhonen et al., 1997). However cleavage of the bovine b-lactoglobulin from the human rEPO restored the biological activity comparable to standard human rEPO (Korhonen et

al., 1997). However fusion-protein-derived rEPO had a lower molecular size of 32.5kd and the transgenic animals had an 80% increase in hematocrit values (Korhonen et al., 1997).

The expression of human erythropoietin in the mammary gland is an attractive cost effective method. The major disadvantage is human rEPO in the milk of transgenic animals resulted in very low expression levels and was detrimental for the founder animals (Toledo et al., 2005). Therefore rEPO has been expressed by direct transduction in mammary epithelium of non transgenic goats (Toledo et al., 2006). Expression levels of human rEPO in milk of non transgenic goats reached 2g/L but were underglycosylated resulting in low *in vivo* hematopoietic activity (Toledo et al., 2006).

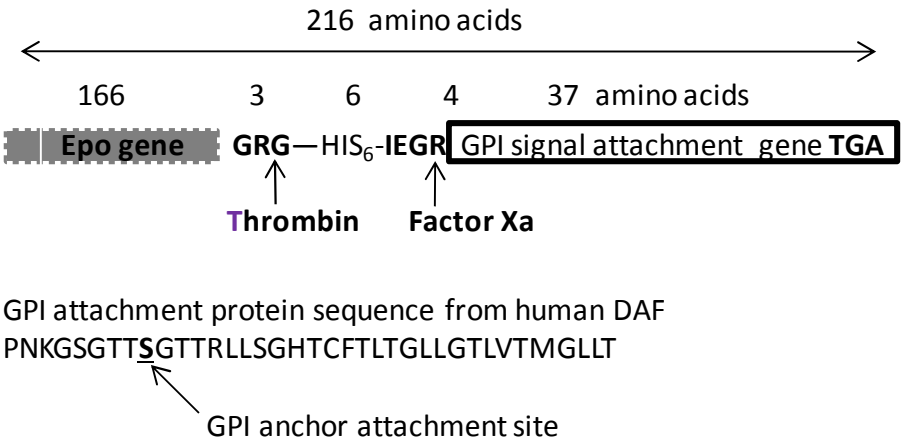

Figure 1: Schematic Representation of the Construct Expressing Human Recombinant EPO (Devasahayam, 2015; Singh et al., 2015). With a His6Tag, a Factor Xa Site and a Thrombin Cleavage Site 5' of the His6Tag. The DAF GPI Attachment Gene Sequence is Shown with the GPI Anchor Attachment Site in Bold

HUMAN rEPO FROM TRANSGENIC PLANTS

Expression of recombinant proteins in transgenic plants has the advantages of low production cost and lower capital investment in infrastructure though not often appropriate post-translational modifications. The major disadvantage of plants as an expression host is the low level of transgene expression. Expression of erythropoietin in potato tuber using patatin, the tuber-tissue-specific promoter for higher expression levels and whole plant from single explants were obtained (Desai and Padh, 2012). Polymerase chain reaction (PCR) analysis was performed to confirm the stable integration of the erythropoietin gene in the potato plant by using sequence-specific primers (Desai PN and Padh H 2012). Human rEPO without sialylation has been expressed along with b-1,4 galactosyltransferase in transgenic tobacco to obtain complex N-glycans. Oligosaccharide analysis gave paucimannosidic, high mannose and complex oligosaccharides (Kittur et al., 2013).

HUMAN rEPO FROM *PICHIA PASTORIS*

Human rEPO expressed from *Pichia pastoris* is a 32-75kd mixture of glycoforms (Teh et al., 2011). rEPO glycofrom variants from *Pichia pastoris* are active *in vivo* (Teh et al., 2011). Recombinant EPO from glycoengineered Pichia pastoris *Pichia pastoris* show complete site occupancy. CHO derived and *Pichia pastoris* derived rEPO show differences in oligosaccharide structures (Gong et al., 2013). Recombinant EPO from *Pichia pastoris* had sialylated biantennary oligosaccharides compared to CHO derived rEPO which have sialylated bi-, tri- and tetra-antennary oligosaccharides (Gong et al., 2013).

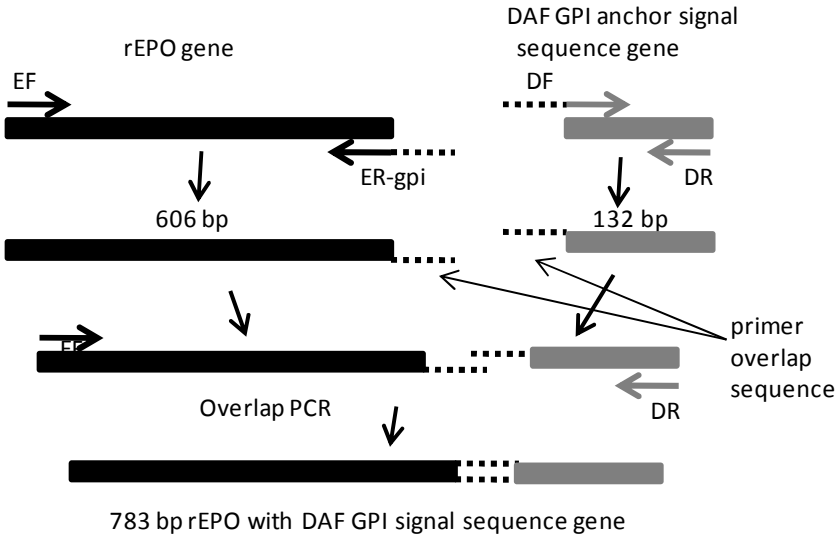

Figure 2: Overlap Extension PCR. rEPO Gene Was Amplified Using Primers EF and ER-gpi; DAF Signal Sequence was Amplified Using DF and DR; the Overlap Primers are ER-gpi and DF. The Two PCR Products of EPO and DF Were Stoichiometrically Combined and the Final rEPO with the DAF Gpi Signal Sequence Gene was Amplified Using Primers EF and DR

HOMOGENOUS RECOMBINANT GLYCOPROTEINS WITHOUT GLYCOSYLATION HETEROGENEITY FROM CHO CELLS

Secretion in terms of expression levels and variable site occupancy was protein dependent as observed in the expression of recombinant soluble Thy-1 in COS-7 cells and CHO-K1 cells (Devasahayam et al., 1999; Devasahayam, 2006). Recombinant GPI anchored Thy-1 in CHO-K1 cells does not show variable site occupancy in that the fully glycosylated form alone was expressed on the cell surface (Devasahayam et al., 1999). The GPI anchored Thy-1 expressed on CHO-K1 cells when purified appeared as a single protein band by SDS-PAGE with no lower molecular weight glycoforms (Devasahayam et al., 1999). Further oligosaccahries from GPI anchored Thy-1 expressed on CHO-K1 cells were similar in structure to human brain derived Thy-1 except in the absence of hybrid oligosaccharides present in brain Thy-1 (Devasahayam et a. 1999). This indicates that (i) the attachment of the protein to the cell membrane increases its stability by presenting the protein to the glycosylation processing enzymes in a protein specific manner resulting in a homogenous glycoprotein population, (ii) glycoforms or unglycosylated protein are efficiently degraded by the cells quality control mechanism leading to the exclusive expression of the fully glycosylated glycoprotein (Devasahayam, 2007).

GPI ANCHORED HUMAN rEPO – FOR GLYCOSYLATION HOMOGENEITY AND COMPLETE GLYCOSYLATION

The ability to produce from CHO cells the 39kd human rEPO alone without any low molecular weight rEPO glycoforms is described below (Devasahayam 2015; Singh et al., 2015; Devasahayam & Singh et al., 2015). This is the same in both molecular weight and the absence of glycoforms to EPO purified from human urine (Miyake et al., 1977). Is is expected that this high molecular weigh rEPO at 39-40kd will have highly processed tetra antennary oligosaccharides required for biological activity (Misaizu et al., 1995) with N-acetyl lactosamine repeats (Sasaki et al., 1987)

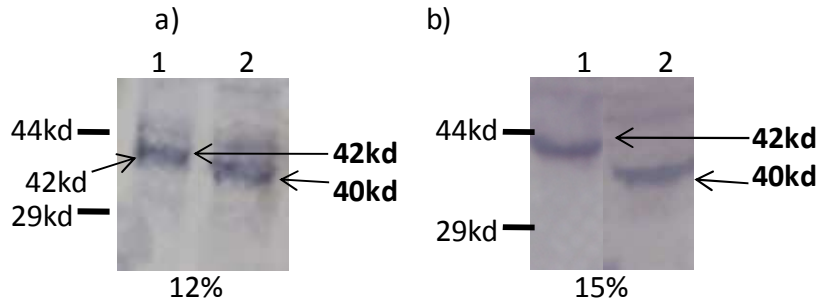

Figure 3: Thrombin Digestion of rEPO-g by Western Blot Using Anti Human EPO Mab (Devasahayam and Singh, 2015) on; a) 12% SDS-PAGE Gel of Undigested (Lane 1) and Thrombin Digested rEPO-g (Lane 2); and b)15% SDS PAGE Gel of Undigested (Lane 1) and Thrombin Digested rEPO-g (Lane 2)

Completely glycosylated human rEPO was obtained when expressed as a GPI anchored molecule in CHO cells with the GPI anchor signal sequence from decay accelerating factor (DAF) (Devasahayam, 2015; Singh et al., 2015) (Figure 1).The GPI anchor signal sequence from DAF was attached to the C-terminal of the EPO gene by overlap extension PCR (Figure 2) and expressed in CHO cells (Singh et al., 2015). Homogenous completely glycosylated human rEPO-GPI anchored at 42kd without low molecular weight glycoform variants was obtained (Figure 3a,b; lane 1) (Devasahayam & Singh, 2015; Singh et al., 2015). The removal of the GPI anchor was designed to include a thrombin cleavage site C terminal to the rEPO gene (Figure 1) (Singh et al., 2015). The 1.8kd 9 amino acid linker region between the rEPO and GPI anchor was removed using thrombin to give fully glycosylated human rEPO at 40kd with an extra GR dipeptide at the c-terminal shown using 12% (Figure 3a lane 2) and 15% discontinuous SDS-PAGE (Figure 3b; lane 2) (Devasahayam and Singh, 2015).

This is the first report demonstrating homogenous and completely glycosylated human rEPO at 40kd from CHO cells relevant for efficient therapy (Devasahayam and Singh, 2015). This completely glycosylated higher molecular weight rEPO at 40kd with possibly tetraantennary N-oligosaccharides and N-acetyl lactosamine repeats will not be filtered from the kidney (Devasahayam, 2015). Therefore rEPO will be maintained at a high plasma for efficient stimulation of erythroid progenitor cells (Misaizu, T. et al. 1995).

CONCLUSIONS

Human recombinant EPO is used extensively to treat anemia and transfusions with a global market estimated at USD 57 billion in 2005. Completely glycosylated and fully processed oligosaccharides are required for biological activity of rEPO. The review describes for the first time the process to obtain homogenous rEPO from CHO cells at a molecular weight of 40kd similar in size to human EPO purified from human urine (Miyake et al. 1977).

REFERENCES

1. Desai, P.N & Padh, H. (2012) Expression of erythropoietin in Indian tetraploid potato variety [v1; ref status: not approved 2, http://f1000r.es/USGpfK] F1000Research 2012, 1:26
2. Devasahayam, M., Catalino, P.D, Rudd, P.M, Dwek, R.A, & Barclay, A.N. (1999) The glycan processing and site occupancy of recombinant Thy-1 is markedly affected by the presence of a glycosylphosphatidylinositol anchor. Glycobiol. 9, 1381-1387

3. Devasahayam, M. (2006) Characterization of glycosylation in COS-7 cells using recombinant soluble Thy-1. Curr Sci. 90, 549-557

4. Devasahayam, M. (2007) Factors affecting the expression of recombinant glycoproteins. Indian J Med Res. 126, 22-27

5. Devasahayam, M. & Singh, P.K. (2015) Comparison of GPI anchored and secreted human recombinant erythropoietin from Chinese hamster ovary cells. Int. Journal of Bio-Technology and Research, 5(3), 27-34

6. Devasahayam, M. (2015) Expression of recombinant glycoproteins with complete N-glycosylation site occupancy from CHO cells. Int J of Bio-Tech and Res. 5, 9-16

7. Gong, B., Burnina, I., Stadheim, T.A, Li, H. (2013) Glycosylation characterization of recombinant human erythropoietin produced in glycoengineered Pichiapastoris by mass spectrometry. J Mass Spectrom. 48, 1308-1317

8. Kausar, H., Gull, S., Ijaz, B., Ahmad, W., Sarwar, M.T., Iqbal, Z., Nawaz, Z., Riazuddin, S., & Hassan, S. (2011) Huh-7 cell line as an alternative cultural model for the production of human like erythropoietin (EPO). J of Translational Med. 9, 186-93.

9. Kingsley, D.M, Kozarsky, K.F, Hobbie, L., & Krieger, M. (1986) Reversible defects in O-linked glycosylation and LDL receptor expression in a UDP-GalNDP-GalNAc 4-epimerase deficient mutant. Cell, 44, 749-759

10. Kittur, F.S., Bah, M., Archer-Hartmann, S., Hung, C.Y., Azadi, P., Ishihara, M., Sane, D.C., Xie, J. (2013) Cytoprotective Effect of Recombinant Human Erythropoietin Produced in Transgenic Tobacco Plants. PLoS ONE 8(10): e76468. doi:10.1371/journal.pone.0076468

11. Korhonen, V., Tolvanen, M., Hyttineni, J., Uusi-Oukari, M., Sinervirta, R., Ahonen, L., Jauhiaainen, M., Janne, O.A & Janne, J. (1997) Expression of bovine P-lactoglobulinhuman erythropoietin fusion protein in the milk of transgenic mice and rabbits. Eur. J. Biochem. 245, 482-489

12. Krieger, M., Reddy, P., Kozarsky, K., Kingsley, D., Hobbie, L., & Penman, M. (1989) Analysis of the synthesis, intracellular sorting, and function of glycoproteins using a mammalian cell mutant with reversible glycosylation defects. Methods Cell Biol. 32, 57-84

13. Misaizu, T., Matsuki, S., Strickland, T.W., Takeuchi, M., Kobata, A., & Takasaki, S. (1995) Role of antennary structure of N-linked sugar chains in renal handling of recombinant human erythropoietin Blood,86, 4097-4104

14. Miyake, T., Kung, C.K., & Goldwasser, E. (1977) Purification of human erythropoietin. J Biol Chem. 252, 5558-5564.

15. Sasaki, H., Bothner, B., Dell, A., & Fukuda, M. (1987) Carbohydrate structure of erythropoietin expressed in Chinese hamster ovary cells by a human erythropoietin cDNA. J Biol Chem. 262, 12059-12076

16. Schriebla, K., Trummera, E., Lattenmayera, C., Weikc, R., Kunertb, R., Müllerb, D., Katingerb, H., & Vorauer-Uhlb, K. (2006) Biochemical characterization of rhEpo-Fc fusion protein expressed in CHO cells. Protein Exp and Purif. 49, 265-275

17. Singh, P.K., Devasahayam, M., & Devi, S. (2014) Expression of GPI anchored human recombinant erythropoietin

in CHO cells is devoid of glycosylation heterogeneity. Ind J of Exp Biol. 53, 195-201.

18. Spivak, J.L, Gascón, P., & Ludwig, H. (2009) Anemia management in oncology and hematology. Oncologist, 1, 43-56. doi: 10.1634/theoncologist.2009-S1-43.
19. Surabattula, R., Rao, K.R.S.S., & Polavarapu, R. (2011) An Optimized Process for Expression, Scale-Up and Purification of Recombinant Erythropoietin Produced in Chinese Hamster Ovary Cell Culture. Res in Biotech. 2, 58-74
20. Teh, S.H, Fong, M.Y., & Mohamed, Z. (2011) Expression and analysis of the glycosylation properties of recombinant human erythropoietin expressed inPichia pastoris. Genet Mol Biol. 34, 464-470
21. Toledo, J.R., Sánchez, O., Montesino, S.R., Fernández, G.Y., Rodríguez, M.P., & Cremata, J.A. (2005) Differential in vitro and in vivo glycosylation of human erythropoietin expressed in adenovirally transduced mouse mammary epithelial cells. Biochim Biophys Acta. 1726, 48-56
22. Toledo, J.R., Sánchez, O., Seguí, R.M., García, G., Montañez, M., Zamora, P.A., Rodríguez, M.P., & Cremata, J.A. (2006) High expression level of recombinant human erythropoietin in the milk of non-transgenic goats. J Biotechnol. 123, 225-235
23. Wasley, L.C., Timony, G., Murtha, P., Stoudemire, J., Dorner, A.J., Caro, J., Krieger, M. & Kaufman, R.J. (1991) The Importance of N- and 0-Linked Oligosaccharides for the Biosynthesis and In Vitro and In Vivo Biologic Activities of Erythropoietin. Blood, 77, 2624-2632
24. Yoon, S.K., Ahn, Y., & Han, K. (2001) Enhancement of recombinant erythropoietin production in CHO cells in an incubator without CO2 addition. Cytotech. 37, 119-132
25. Zhang, P., Chan, K.F., Haryadi, R., Bardor, M. & Song, Z. (2013) CHO Glycosylation Mutants as Potential Host Cells to Produce Therapeutic Proteins with Enhanced Efficacy. Adv Biochem Eng Biotechnol. 131, 63–87
26. Zoph, D. & Vergis, G. (2002) Glycosylation: a critical issue in protein development and manufacturing. Pharm Visions, 10-14